Emergence of a new swine H3N2 and pandemic (H1N1) 2009 influenza A virus reassortant in two Canadian animal populations, mink and swine

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ABSTRACT

A swine H3N2 (swH3N2) and pandemic (H1N1) 2009 (pH1N1) influenza A virus reassortant (swH3N2/pH1N1) was detected in Canadian swine at the end of 2010. Simultaneously, a similar virus was also detected in Canadian mink based on partial viral genome sequencing. The origin of the new swH3N2/pH1N1 viral genes was related to the North American swH3N2 triple reassortant cluster IV (for HA and NA genes) and to pH1N1 for all the other genes (M, NP, NS, PB1, PB2, PA). Data indicate that the swH3N2/pH1N1 can be found in several pigs that are housed at different locations.

Influenza (flu), is a disease affecting several animal species including human. In our recent history, several pandemics arise in human population which were estimated to cause millions of death such as Spanish flu (1918-20), Asian Flu (1957-58) and Hong Kong Flu (1968-69). Its etiological agent, influenza A virus, belongs to the virus family *Orthomyxoviridae*. Influenza A virus is a single-stranded RNA virus with a segmented genome composed of eight gene segments now recognized to encode for 12 protein products (21). These are the polymerase basic protein 2 encoded by segment 1 (PB2); PB1, PB1-F2, and N40 proteins encoded by segment 2 (PB1); polymerase acidic protein encoded by segment 3 (PA); hemagglutinin protein encoded by segment 4 (HA); nucleoprotein encoded by segment 5 (NP); neuraminidase protein encoded by segment 6 (NA); matrix (M1) and ion channel (M2) proteins encoded by segment 7 (M) and 2 nonstructural proteins encoded by segment 8 (NS). Sixteen HA and 9 NA antigenic types have been reported so far and are used to classify viruses into subtypes H1 to H16 and N1 to N9 (4). Genetic diversity in influenza virus results from a high replication error rate associated with low-fidelity RNA polymerase, and the reassortment of gene segments among coinfecting strains. In 1998, an H3N2 triple reassortant influenza A virus emerged in the U.S. swine population (10, 19, 22). RNA viral genes were identified as a mix of classical swine, human and avian virus lineages. In 2005, spreading of this virus was seen in the swine, turkey and two years later in mink populations of Canada (5, 15). Today, the influenza A virus subtypes that are mostly found in swine in Canada are usually H1N1 and H3N2 (13, 14). Other subtypes, such as H1N2, H3N3, and H4N6, are also sporadically found (8, 9, 11). In April 2009, a novel pandemic triple-reassortant influenza A H1N1 (pH1N1) virus was detected and spread globally as the first influenza pandemic

60 of the 21st century. Role of the animals in the spreading of the pH1N1 is still unknown,
61 but several reports showed that human to animal transmission of the virus have occurred
62 (1, 7). Now, pH1N1 is frequently found in Canadian swine population(14). Herein, the
63 emergence of a new swine H3N2 (swH3N2) and pH1N1 reassortant (swH3N2/pH1N1) in
64 two Canadian animal populations, mink and swine, is reported.
65
66 In December 2010, an outbreak of respiratory symptoms occurred in a hog farm
67 located in the province of Quebec, Canada. At the site, about 10% of the piglets were
68 coughing, sows had no clinical sign and several fattening pigs were coughing with nasal
69 discharge. No increase of death rate was observed during this swine flu episode compared
70 to the death rate that was occurring within the herd prior to the outbreak. Pools of nasal
71 swabs of sick fattening pigs were collected at the farm and submitted to test for the
72 presence of swine influenza virus (SIV) by a real-time reverse transcriptase PCR (RT-
73 PCR) which is able to detect influenza A viruses by targeting the conserved region of the
74 M gene (17, 20). In addition, RT-PCR assays which are specific for the HA and NA most
75 common subtypes found in swine (H3 versus H1; N2 versus N1) were conducted (2).
76 Even if the general Influenza A virus PCR assay gave a low positive response, the case
77 was highly positive for the H3 and N2 subtypes
78 (A/swine/Quebec/1259260/2010(H3N2)). For the reconciliation of the obtained results,
79 sequencing of the HA, M and NS genes was conducted. Sequence analyses revealed a
80 new commingling of the HA, M and NS genes subtypes in a single swine case (data not
81 shown). To further confirm the appearance in swine population of a new influenza virus
82 reassortant, a small retrospective study was conducted. Thus, the M gene of all swH3N2

PCR positive cases (n = 13 cases) that were submitted to the Molecular diagnostic laboratory (MDL) of the University of Montreal from October 2010 to February 2011, were sequenced. All those cases originated from different herds experiencing swine flu episode. Those herds were located at different sites across the province of Quebec, Canada. For those cases, various clinical samples were submitted, such as lung tissues and/or nasal swabs. When sequence analyses revealed that the M genes were genetically related to pH1N1, then virus isolation was conducted. Overall, the sequence analyses of the M gene PCR products revealed that 9 cases, over the 13 swH3N2 PCR positive cases, possessed a new reassortment of the HA, NA and M genes (Figure 1), the M originating from the pH1N1 strain (Figure 1). The M gene of the remaining 4 cases was genetically related to swH3N2 (data not shown). The earliest confirmed submitted case of the new influenzavirus reassortant was obtained in October 2010.

Specific pathogen free 10 days old embryonated chicken eggs were inoculated via the chorioallantoic sac for virus isolation as previously described (5). The presence of the influenza virus within the allantoic fluid collected at the second and/or third passage was confirmed by hemagglutination, transmission electron microscopy and PCR assays. The viral RNA was isolated from submitted samples and allantoic fluids using a commercial kit (QIAamp Viral RNA Mini Kit, Qiagen, Mississauga, Ontario). The full-length of the viral RNA segments were amplified by reverse transcription-PCR (RT-PCR), the PCR products were purified (QIAquick PCR purification kit; Qiagen) and both strands of the purified DNA PCR products were sequenced using the same primer sets used in the RT-PCR reaction with standard automated sequencing methods, as previously described (5).

106 Resulting sequences were compared with other SIV reference sequences such as those
107 available in the Diagnostic Veterinary Virology Laboratory (DVVL) of the University of
108 Montreal (5) and in GenBank database. BioEdit Sequence Alignment Editor version
109 7.0.9, (Ibis Therapeutics; Carlsbad, California, USA) and Geneious bioinformatics
110 version 5.4.6 (Biomatters Ltd; Auckland, New Zealand) were used for sequence analyses
111 and the phylogenic tree construction as previously described (5). From the 9 cases
112 confirmed to possess pH1N1 M gene, 6 influenzavirus strains were isolated:
113 A/swine/Quebec/1265553/2010(H3N2) (sw/Qc/1265553/10),
114 A/swine/Quebec/1262080/2010(H3N2) (sw/Qc/1262080/10),
115 A/swine/Quebec/1267568/2010(H3N2) (sw/Qc/1267568/10),
116 A/swine/Quebec/1257774/2010(H3N2) (sw/Qc/1257774/10),
117 A/swine/Quebec/1257777/2010(H3N2) (sw/Qc/1257777/10),
118 A/swine/Quebec/1267566/2011(H3N2) (sw/Qc/1267566/11). Noteworthy, 5 isolates
119 were tested in cell culture and were confirmed to be able to replicate in MDCK (Madin-
120 Darby canine kidney) cell line. Subsequently, the viral genomes of the 6 isolated strains
121 were sequenced and analysed (Figure 1). Overall, the viral genes of the new
122 influenzavirus reassortant (swH3N2/pH1N1) are genetically related to swH3N2 for the
123 HA and NA genes and pH1N1 for the M, NP, NS, PB1, PB2 and PA genes (Figure 1).
124 The nucleotide sequence identities of the swH3N2/pH1N1 viral genes compared to their
125 respective swH3N2 (A/swine/Quebec/4001/2005(H3N2)) and pH1N1 (A/New
126 York/19/2009(H1N1)) reference strains counterpart varied between 95.6-97.5%, 97.2-
127 98.4%, 98.7-99.8%, 98.8-99.5%, 99.2-99.7%, 97.3-99.7%, 99.3-99.7% and 99.2-99.5%
128 for the HA, NA, M, NS, NP, PB1, PB2 and PA genes, respectively (Figure 1). As

129 illustrated in Figure 2, the HA genes of the 6 swH3N2/pH1N1 strains classified within
130 the swH3N2 triple reassortant cluster IV (Figure 2). Noteworthy, the swH3N2/pH1N1
131 strains seem to cluster within two subgroups named subcluster IVa and IVb (Figure 2).
132 The HA pairwise nucleotide (nt) identities between the two swH3N2/pH1N1 subclusters
133 were 94.7-95.9% and within each subclusters were 96.4-98% and 99.1-99.8% for IVa and
134 IVb, respectively. Overall, the HA amino acids identities between the swH3N2/pH1N1
135 strains were 94.5-99.8% (data not shown), which suggested the existence of possible
136 antigenic variations between those strains.
137
138 To ascertain this hypothesis, an hemagglutination inhibition assays (HI) was
139 conducted, as previously described (5), using as antigen the 6 swH3N2/pH1N1 isolated
140 strains. The reference sera used in the HI assay, ck/150/90 and sw/4001/05, were
141 obtained following immunization of chicks with a formalin-inactivated H3N2 SIV strain
142 that was isolated in Quebec in 1990 or from naturally infected pig with the reference
143 strain A/Swine/Quebec/4001/05 (H3N2) (5), respectively. In addition, 20 sera collected
144 from 6 to 8 weeks old pigs originating from the barn where animals have experienced the
145 first confirmed flu case related to the swH3N2/pH1N1 reassortant (case:
146 A/swine/Quebec/1259260/2010, which is a strain classified within subcluster IVa as
147 illustrated in Figure 2) were tested. Statistical analyses were realized using the GraphPad
148 Prism version 4 software. The one-way ANOVA non-parametric Friedman test was used
149 to determine if there is statistical significant difference between viruses in regards to the
150 HI results. Interestingly, the antigenic reactivity of the 20 pig sera tested was
151 significantly higher ($p < 0.05$) against homologous strains of subcluster IVa compared to

152 heterologous strains of subclusters IVb (Figure 3). In addition, the HI titers of the
153 reference sera (ck/150/90 and sw/4001/05) demonstrated the existence of antigenic
154 differences between IVa and IVb strains (Figure 3). Overall, the HI results confirmed the
155 existence of antigenic variations between strains of subclusters IVa and IVb (Figure 3)
156 supporting the genomic results (Figure 2).
157
158 Concomitant to the characterization of the first swine flu case, RNA extracted
159 from a mink tissue sample sent by the provincial laboratory of Nova Scotia, Canada, were
160 received for further characterization. The strain (A/mink/Nova Scotia/1265707/2010) was
161 confirmed to be a H3N2 strain by PCR and originated from a farm where mink kits were
162 presenting serious coughing but very low mortality. The HA gene of the new mink strain
163 shared higher nt homologies with the 6 swH3N2/pH1N1 strains and a swH3N2 reference
164 strain (95.7% to 100%) compared to pH1N1 (52.6%) (Figure 1 and 2). Further
165 sequencing of the mink strain M gene showed a 99.8% nt homology to pH1N1 (Figure 1)
166 and only 88% compared to swH3N2. Unfortunately, not enough tissue was available for
167 further characterization and virus isolation.
168
169 The characterization of the swH3N2/pH1N1 reassortant viruses from swine in the
170 province of Quebec indicates that reassortment of gene segments has occurred between
171 the North-American swine H3N2 triple-reassortant from cluster IV and the pH1N1
172 strains as earlier as October 2010. Virus reassortants possess two genes related to
173 swH3N2 (HA and NA) and all others to the pH1N1. The fact that 6 swH3N2/pH1N1
174 strains were isolated in a short period of time from animals with clinical signs housed at

different locations suggests an active transmission of this virus reassortant. Since a similar strain was also found in a sample recovered from a mink, then this could illustrate the potential of the virus to cross the species barrier. How and when the pig and mink populations of Canada acquired the new swH3N2/pH1N1 strain is unknown. Noteworthy, Gagnon et al. (2009) have reported that minks are fed with uncooked pork by-products coming from slaughterhouse facilities (i.e. discarded tissues: such as lung which is the target of SIV) (Figure 1) (5). This could explain how the cross-species contamination arises. Further studies will have to be conducted to establish the full nature of the swH3N2/pH1N1 in the mink population.

Ancestral genes present in the pH1N1 virus suggest that the virus has been circulating undetected in the swine population for an undetermined period of time. This event underlines a need of the surveillance of influenza viruses in the swine populations (6). In our diagnostic laboratory, as seen in many others, routine surveillance of influenza viruses in swine is done by detection of Influenza A virus by targeting the M gene. Further characterization is often performed by typing the HA and NA genes. Such a procedure is not sufficient to differentiate reassortants explaining why the new virus has been unnoticed by the regular surveillance. It is likely that more cases are expected to be positive as surveillance programs adapt their testing. Concerns should also be raised about the surveillance of human seasonal influenza A (H3N2). For example, inoculation in ferrets with a reassortant pH1N1 and seasonal human influenza A (H3N2) NA showed increased severity of lesions probably related to a higher viral replication (16).

198 Reassortant viruses originating from the pH1N1, different from the
199 swH3N2/pH1N1 of the present manuscript, have already been described in swine from
200 around the world in the year 2010 (3, 12, 18) raising the concern of a possibly more
201 pathogenic reassortant. Most of the pH1N1 and SIV reassortants that have been reported
202 are H1N2 viruses (3, 12). Interestingly, one other swine H3N2 strain
203 (sw/MN/239105/09), which possesses pH1N1 related genes, has been recently reported in
204 United States (3). However, the genes profile of sw/MN/239105/09 is different compared
205 to our swH3N2/pH1N1 strains suggesting at least two reassortment events over time. In
206 infected ferrets, the virulence of sw/MN/239105/09 strain was similar compared to a
207 North American swH3N2 triple reassortant reference strain (3). Similarly, clinical
208 observations in the field also indicate that the swH3N2/pH1N1 strains are not more
209 pathogenic than circulating endemic strains. Nonetheless, full genome sequencing of SIV
210 should be regularly performed and the strains pathogenicity should be closely monitored.
211

Nucleotide sequence Genbank accession numbers. A/swine/Quebec/1259260/2010(H3N2): JF420890, JF420891 and JF411012; A/swine/Quebec/1265553/2010(H3N2): JF682719-JF682724, JF703684 and JF703685; A/swine/Quebec/1262080/2010(H3N2): JN584176-JN584182 and JN817421; A/swine/Quebec/1267568/2010(H3N2): JN584183-JN584190; A/swine/Quebec/1257774/2010(H3N2): JN584191-JN584198; A/swine/Quebec/1257777/2010(H3N2): JN584199-JN584206; A/swine/Quebec/1267566/2011(H3N2): JN584207-JN584214; A/mink/Nova Scotia/1265707/2010(H3N2): JF682717 and JF682718.

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FIGURE LEGENDS

Figure 1. Viral genome sequences homologies of the new swH3N2/pH1N1 reassortant virus with swH3N2 and pH1N1 reference strains. When two squares between a swH3N2/pH1N1 strain and a reference strain possess the same color, it indicates that the viral gene is genetically related. Influenza virus reference strains used for comparison are A/New York/19/2009(H1N1) (Genbank: FJ984388-FJ984394, GQ457503) and the swH3N2 cluster IV Quebec reference strain A/swine/Quebec/4001/2005(H3N2) (Genbank: EU826543-EU826550). Numbers in squares indicate the % of nucleotide (nt) homology compared to the genetically related strain. n.d.: not determined.

Figure 2. Phylogenetic tree of the HA gene nucleotide sequences of the swH3N2/pH1N1 strains. The designated clusters I, II, III and IV classifies the North-American H3N2 triple-reassortant swine viruses as previously described (4). Phylogenetic tree was estimated by the Neighbor-Joining method using the Geneious v5.4.6 software with a bootstrap resampling method (1000 replications). Bootstrap confidence levels are indicated at the nodes of the phylogenetic tree. Bootstrap values lower than 50 were not indicated for clarity of the figure. GenBank accession numbers for the sequences of all viruses are provided after their names. Horizontal scale indicates the distances between strains; a 0.02 distance means that the strains possess 98% nucleotide identity. Asterisk and triangle indicate the swine and mink swH3N2/pH1N1 strains, respectively, identified in this study. Those strains have been classified within

subclusters IVa and IVb. The arrow indicates the other swH3N2 and pH1N1 reassortant strain that has been recently reported in United States by Ducatez et al. (2011)(3), its genes are related to swH3N2 (for HA, NA, NS, PB1 and PB2) and pH1N1 (for M, PA and NP).

Figure 3. Hemagglutination inhibition (HI) titers of convalescence pig sera toward swH3N2/pH1N1 viruses. Twenty sera collected from 6 to 8 weeks old pigs housed with the animal experiencing the first confirmed flu case related to the swH3N2/pH1N1 reassortant (case: A/swine/Quebec/1259260/2010) were tested. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers extending below and above the box show the lowest and highest HI values obtained with the 20 pig sera that were tested. When a HI result was negative at the lowest serum dilution (1/10) tested, the value of the HI titer was established to be equal to 0. When 2 sets of data are labelled with superscripts of different letters it indicates that these 2 sets of data are statistically different ($P < 0.05$). Below the X axis, a table indicates the HI values of the reference sera (ck/150/90 and sw/4001/05) against each SIV strains tested.

Figure 1

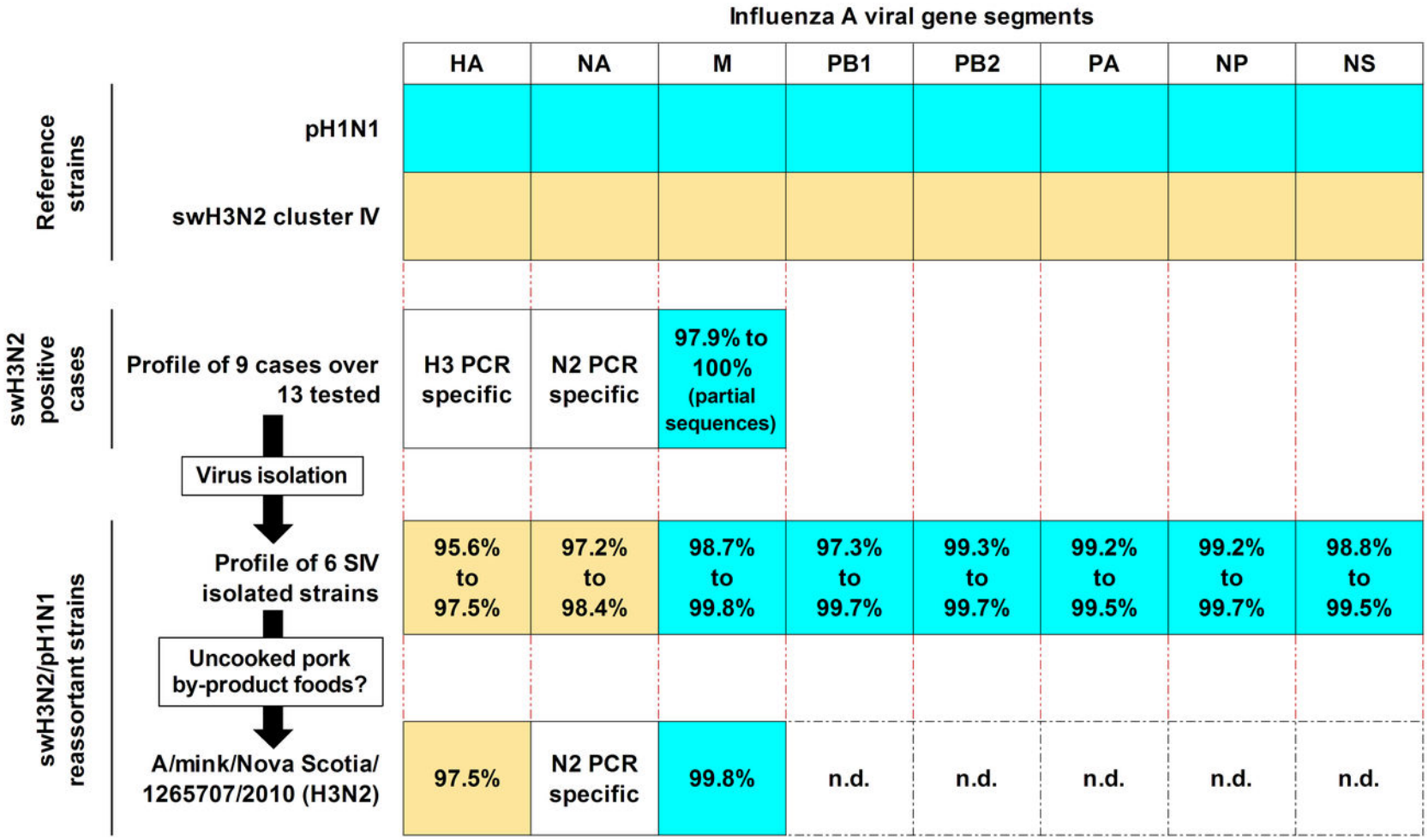

Influenza A viral gene segments
HA
NA
M
PB1
PB2
PA
NP
NS
Reference strains
pH1N1
swH3N2 cluster IV
swH3N2 positive cases
Profile of 9 cases over 13 tested
H3 PCR specific
N2 PCR specific
97.9% to 100% (partial sequences)
Virus isolation
swH3N2/pH1N1 reassortant strains
Profile of 6 SIV isolated strains
95.6% to 97.5%
97.2% to 98.4%
98.7% to 99.8%
97.3% to 99.7%
99.3% to 99.7%
99.2% to 99.5%
99.2% to 99.7%
98.8% to 99.5%
Uncooked pork by-product foods?
A/mink/Nova Scotia/ 1265707/2010 (H3N2)
97.5%
N2 PCR specific
99.8%
n.d.
n.d.
n.d.
n.d.
n.d.

Figure 2

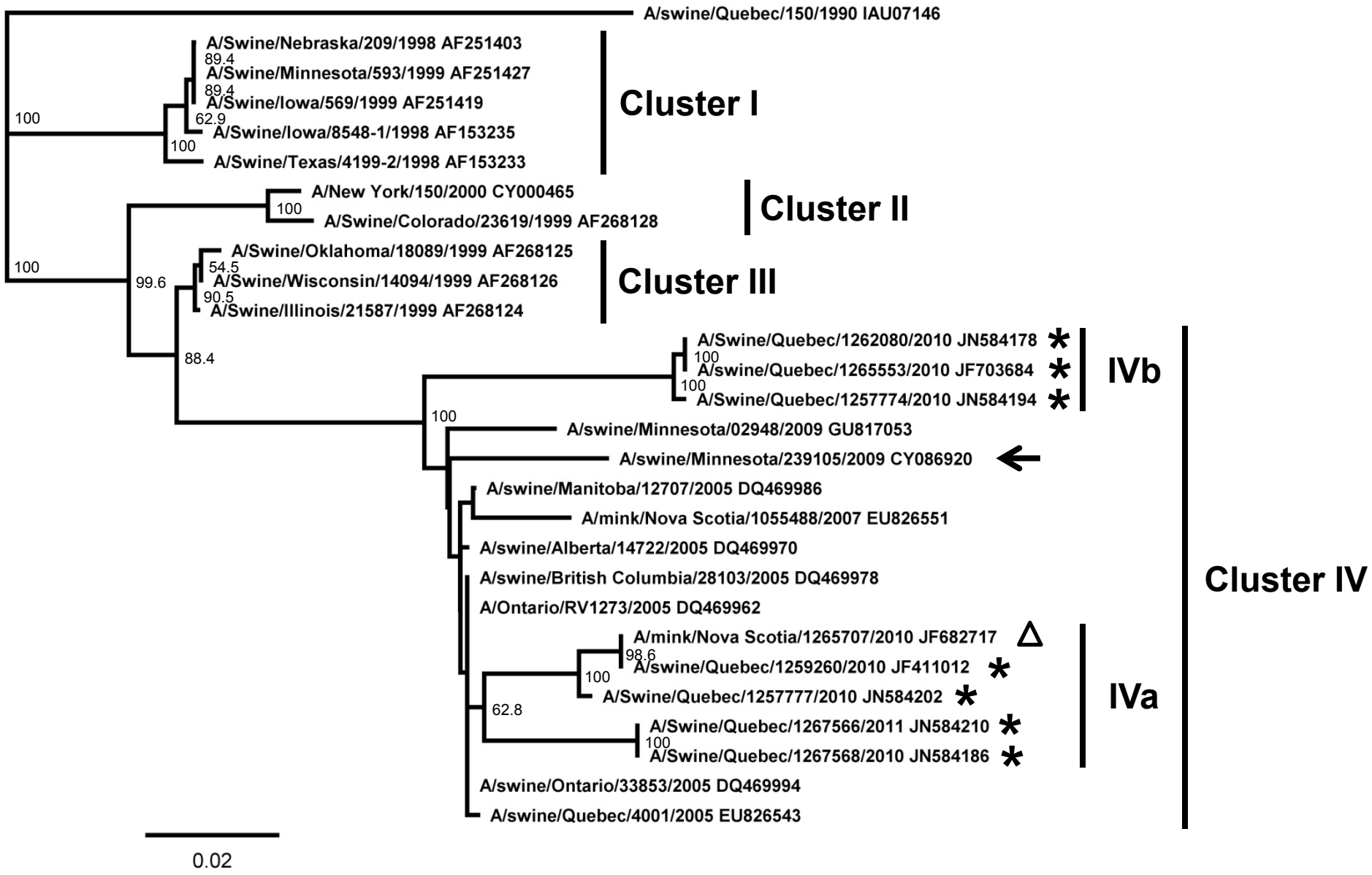
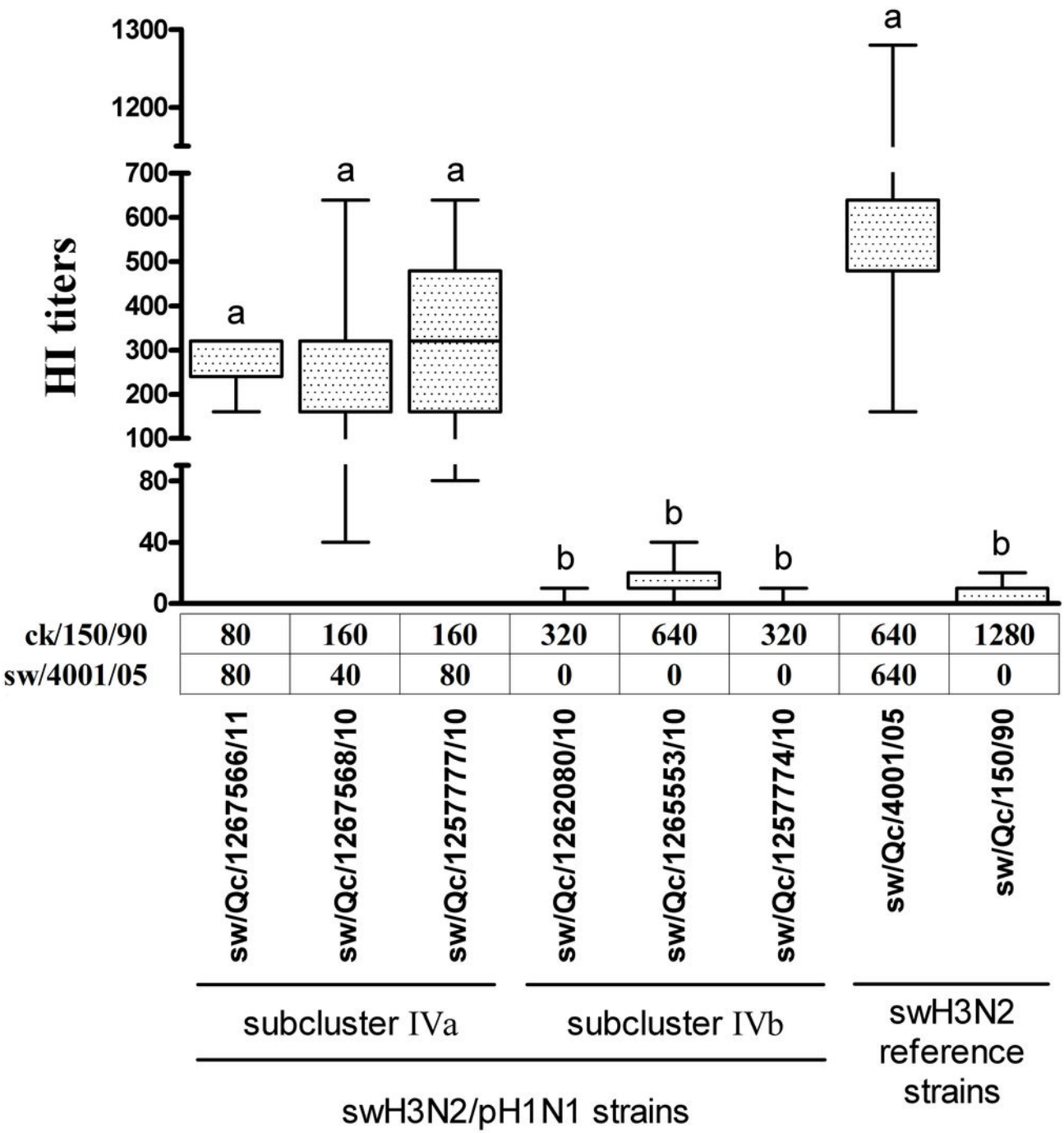

A/swine/Quebec/150/1990 IAU07146
A/Swine/Nebraska/209/1998 AF251403
89.4
A/Swine/Minnesota/593/1999 AF251427
89.4
A/Swine/Iowa/569/1999 AF251419
62.9
A/Swine/Iowa/8548-1/1998 AF153235
100
A/Swine/Texas/4199-2/1998 AF153233
100
Cluster I
A/New York/150/2000 CY000465
100
A/Swine/Colorado/23619/1999 AF268128
Cluster II
A/Swine/Oklahoma/18089/1999 AF268125
54.5
A/Swine/Wisconsin/14094/1999 AF268126
90.5
A/Swine/Illinois/21587/1999 AF268124
100
99.6
Cluster III
A/Swine/Quebec/1262080/2010 JN584178 *
100
A/swine/Quebec/1265553/2010 JF703684 *
100
A/Swine/Quebec/1257774/2010 JN584194 *
IVb
88.4
100
A/swine/Minnesota/02948/2009 GU817053
A/swine/Minnesota/239105/2009 CY086920
A/swine/Manitoba/12707/2005 DQ469986
A/mink/Nova Scotia/1055488/2007 EU826551
A/swine/Alberta/14722/2005 DQ469970
A/swine/British Columbia/28103/2005 DQ469978
A/Ontario/RV1273/2005 DQ469962
A/mink/Nova Scotia/1265707/2010 JF682717 Δ
98.6
A/swine/Quebec/1259260/2010 JF411012 *
100
A/Swine/Quebec/1257777/2010 JN584202 *
62.8
A/Swine/Quebec/1267566/2011 JN584210 *
100
A/Swine/Quebec/1267568/2010 JN584186 *
IVa
A/swine/Ontario/33853/2005 DQ469994
A/swine/Quebec/4001/2005 EU826543
Cluster IV
0.02

Figure 3


HI titers
1300
1200
700
600
500
400
300
200
100
80
40
0
a
a
a
b
b
b
a
b
ck/150/90 80 160 160 320 640 320 640 1280
sw/4001/05 80 40 80 0 0 0 640 0
sw/Qc/1267566/11
sw/Qc/1267568/10
sw/Qc/1257777/10
sw/Qc/1262080/10
sw/Qc/1265553/10
sw/Qc/1257774/10
sw/Qc/4001/05
sw/Qc/150/90
subcluster IVa
subcluster IVb
swH3N2 reference strains
swH3N2/pH1N1 strains